

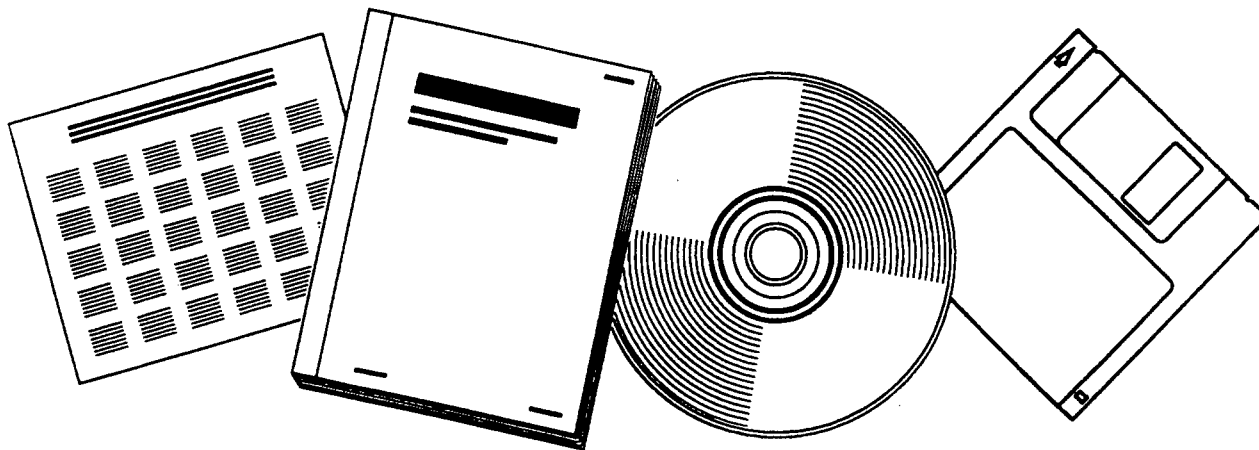


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BIODEGRADATION OF TRIHALOMETHANES AND OTHER HALOGENATED ALIPHATIC COMPOUNDS

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HALOGENATED ALIPHATIC COMPOUNDS**

WRRRI Technical Completion Report No. 298

Geoffrey B. Smith

NEW MEXICO WATER RESOURCES RESEARCH INSTITUTE
New Mexico State University
Box 30001, Dept. 3167
Las Cruces, New Mexico 88003-0001
Telephone (505) 646-4337 FAX (505) 646-6418

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By

Geoffrey B. Smith
Principal Investigator
Department of Biology
New Mexico State University

TECHNICAL COMPLETION REPORT

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New Mexico Water Resources Research Institute

in cooperation with

Department of Biology
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16. Abstract <p>The biological dehalogenation of common water pollutants such as trichloromethane (chloroform) and other halogenated aliphatic compounds was the subject of this project. Samples from diverse water environments such as from groundwater contaminated with halogenated compounds and wastewater from regional treatment plants were studied to identify microorganisms in samples from a secondary anaerobic digester were able to dechlorinate trichloromethane but not dichloromethane (DCM) and samples from a denitrification tank were able to dechlorinate DCM but not trichloromethane. Gene probe analyses of DNA extracted from the dichloromethane-degrading wastewater indicated the presence of the gene coding for dichloromethane dehalogenase, indicating the genetic basis for the dechlorination activity observed. The chloroform dechlorination activity observed in the digester samples was inhibited by the methanogenesis inhibitor bromoethanesulfonic acid (BES), and this inhibition could be reversed by coenzyme M (CoM), a coenzyme unique to methanogenic bacteria. These studies indicate that methanogenic bacteria are the organisms responsible for the chloroform dechlorination. Interestingly, very low levels of chloroform (ca. 10 ppb) potentially inhibited methane production in the samples, yet when the chloroform was converted to dichloromethane, methanogenesis resumed quickly. The chloroform dechlorination thus appears to be a means of detoxification for the methanogens, and we propose that the site where chloroform inhibits methanogenesis is also the site by which it is reduced and detoxified to dichloromethane. Applying the chloroform and DCM activities resulted in the design of a 2-stage anaerobic bioreactor which completely dehalogenated chloroform stepwise to DCM, CO_2, and microbial biomass.</p> <p>Dechlorination of a common chlorofluorocarbon (CFC-11) was identified in samples taken from a regional aquifer contaminated with halogenated aliphatic compounds. We have shown that sulfate-reducing bacteria were responsible for this activity and that the process depended on both an electron donor (2-4 carbon fatty acids) and an electron acceptor (sulfate, but not thiosulfate or elemental sulfur). Dechlorination by these aquifer bacteria exhibited an unusual kinetic response in that the reaction was inhibited at either very low (ca. 10 ppb) or relatively high (ca. 10 ppm) concentrations of CFC-11 (the aquifer was contaminated with 0.1 - 2 ppm CFC-11). Calculations of thermodynamic parameters such as the change in free energy and the reducing potential associated with the CFC dehalogenation reactions has given us a predictive understanding as to why one reaction is favored over another. For example, dechlorination of CFC-11 is energetically more favorable than defluorination, and CFC dechlorination was the reaction observed to occur. Such correlations between the predicted and the observed pathway of dehalogenation can be used in feasibility decisions concerning bioremediation of waters contaminated with halogenated aliphatic compounds.</p>		
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TABLE OF CONTENTS

DISCLAIMER	ii
ACKNOWLEDGEMENTS	iii
LIST OF FIGURES AND TABLES	v
ABSTRACT	vi
JUSTIFICATION	1
METHODS	2
RESULTS AND SIGNIFICANCE	4
Biological Dechlorination of Chloroform to Dichloromethane	4
Dichloromethane Degradation--Correlation of Gene Probe and Activity Assays	8
Design and Function of a Two-stage Anaerobic Bioreactor to Degrade Chloroform	12
Dehalogenation of Chlorofluorocarbons (CFCs) by Aquifer Bacteria	14
Thermodynamic Parameters Used to Predict Dehalogenation	17
PRINCIPAL FINDINGS, CONCLUSIONS AND RECOMMENDATIONS	18
SUMMARY	20
REFERENCES	21

LIST OF FIGURES AND TABLES

Figure 1. Reductive Dechlorination of Chloroform	5
Figure 2. Chloroform Dechlorination and Methanogenesis	6
Figure 3. Dechlorination and Methanogenesis Inhibition by BES	7
Figure 4. Reversal of BES Inhibition by Coenzyme M	7
Figure 5. Dichloromethane Degradation by <i>Hyphomicrobium</i> and Wastewater Microbes	9
Figure 6. <i>Hyphomicrobium</i> and Wastewater Microbe DNA Hybridization Patterns	11
Figure 7. Two-stage Anaerobic Bioreactor Performance in Degrading Chloroform	13
Figure 8. CFC Biodegradation Associated With Cellular Fraction	15
Figure 9. CFC Degradation as Affected by CFC-11 Concentration	16
Table 1. Biological Dechlorination as Affected by Reaction Reduction Potential	18
Table 2. Summary of Biological Dehalogenation Reactions	20

ABSTRACT

The biological dehalogenation of common water pollutants such as trichloromethane (chloroform) and other halogenated aliphatic compounds was the subject of this project. Samples from diverse water environments such as from groundwater contaminated with halogenated compounds and wastewaters from regional treatment plants were studied to identify conditions that favor certain dehalogenation reactions over others. For example, wastewater microorganisms in samples from a secondary anaerobic digester were able to dechlorinate trichloromethane but not dichloromethane (DCM) and samples from a denitrification tank were able to dechlorinate DCM but not trichloromethane. Gene probe analyses of DNA extracted from the dichloromethane-degrading wastewater indicated the presence of the gene coding for dichloromethane dehalogenase, indicating the genetic basis for the dechlorination activity observed. The chloroform dechlorination activity observed in the digester samples was inhibited by the methanogenesis inhibitor bromoethanesulfonic acid (BES), and this inhibition could be reversed by coenzyme M (CoM), a coenzyme unique to methanogenic bacteria. These studies indicate that methanogenic bacteria are the organisms responsible for the chloroform dechlorination. Interestingly, very low levels of chloroform (ca. 10 ppb) potentially inhibited methane production in these sample, yet when the chloroform was converted to dichloromethane, methanogenesis resumed quickly. The chloroform dechlorination thus appears to be a means of detoxification for the methanogens, and we propose that the site where chloroform inhibits methanogenesis is also the site by which it is reduced and detoxified to dichloromethane. Applying the chloroform and DCM activities resulted in the design of a 2-stage anaerobic bioreactor which completely dehalogenated chloroform stepwise to DCM, CO₂ and microbial biomass.

Dechlorination of a common chlorofluorocarbon (CFC-11) was identified in samples taken from a regional aquifer contaminated with halogenated aliphatic compounds. We have shown that sulfate-reducing bacteria were responsible for this activity and that the process depended on both an electron donor (2-4 carbon fatty acids) and an electron acceptor (sulfate, but not thiosulfate or elemental sulfur). Dechlorination by these aquifer bacteria exhibited an unusual kinetic response in that the reaction was inhibited at either very low (ca. 10 ppb) or relatively high (ca. 10 ppm) concentrations of CFC-11 (the aquifer was contaminated with 0.1 - 2 ppm CFC-11). Calculations of thermodynamic parameters such as the change in free energy and the reducing potential associated with the CFC dehalogenation reactions has given us a predictive understanding as to why one reaction is favored over another. For example, dechlorination of CFC-11 is energetically more favorable than defluorination, and CFC dechlorination was the reaction observed to occur. Such correlations between the predicted and the observed pathway of dehalogenation can be used in feasibility decisions concerning bioremediation of waters contaminated with halogenated aliphatic compounds.

Keywords: bioremediation, halogenated aliphatic compounds, chlorofluorocarbons (CFCs), dehalogenation, groundwater, wastewater, anaerobic, bacteria.

JUSTIFICATION

The objective of this research was to screen samples (from groundwater, wastewater and pure cultures of bacteria) for biological dehalogenation activity and to identify the metabolic and genetic bases of these processes. The application of the research was to improve our understanding of the dehalogenation of common groundwater pollutants such as the trihalomethanes (THMs, such as chloroform), dichloromethane and the chlorofluorocarbons (CFCs, such as CFC-11 and 113) in order to exploit these processes to remediate or prevent water contamination by halogenated aliphatic compounds.

We have documented the dehalogenation of chloroform, CFC-11 and dichloromethane under three conditions of varying redox potential: methanogenic, sulfate-reducing and denitrifying, respectively. The three water environments in which these activities were identified were a wastewater digester (Yu and Smith, 1996), an aquifer contaminated with halogenated aliphatic compounds (Sonier et al. 1994) and a denitrifying wastewater tank (Melendez et al. 1993; Yu et al. 1996). Calculating thermodynamic parameters of these reactions has proven to be a helpful framework to integrate these reactions and predict which dechlorination reactions are favored under what conditions. Such a predictive understanding of biological dehalogenation has resulted from a recent collaboration with Dr. Jan Dolting (of the Research Institute for Agrobiology and Soil Fertility, Haren, The Netherlands) in which we correlate the dechlorination reactions observed to the changes in Gibbs free energy and redox of the reactions. Such correlations have shown that there may be a critical redox potential which can be used to predict whether certain dehalogenation reactions will occur (Dolting and Smith, manuscript in preparation).

We requested and were granted a six-month extension of our Water Resources Research Institute (WRRRI) project in order to apply some of our results to developing a two-stage bioreactor to mineralize chloroform without the need to aerate the second stage. We have developed this bioreactor so that the methanogenic wastewater sample dechlorinates chloroform

to dichloromethane, which is fed to a second column which mineralizes this to CO₂ and inorganic chloride under denitrifying conditions (Patent Application 2/96; Yu and Smith, 1996b). This two-stage anaerobic bioreactor thus mineralizes more than 99% of the influent chloroform. Taken together, results have aided our predictive understanding of biological dehalogenation in terms of identifying the redox conditions favoring certain biodegradative reactions over others, as well as given us the opportunity to apply two of these reactions to detoxify the most common THM, chloroform.

METHODS

Gas Chromatography. The headspace concentrations of chloroform, CFC-11 and DCM were analyzed by a gas chromatograph (Hewlett Packard, 5890 Series II) equipped with an HP-5 capillary column (25m x 0.32mm x 0.52μm, Hewlett Packard) and an electron capture detector (ECD). The concentrations reported here are in terms of the total mass measured per liquid volume present in the vials, that is the nominal aqueous concentrations. Using Henry's Constants to calculate the amount actually present in aqueous solution results in 1 ppm nominal concentration = 0.18 ppm CFC-11, 0.85 ppm chloroform and 0.91 ppm dichloromethane (Mackay and Shiu, 1981; Gossett, 1987). The halogenated compound concentrations were determined from headspace standards analyzed in the same way and had detection limits of between 1.0 and 10.0 ppb nominal concentrations.

Methane production was measured with a GC equipped with a Nukol capillary column (15m x 0.53mm x 0.50μm, Supelco) and a flame ionization detector (FID). Methane standard curves were used to quantify the methane levels in the sample headspace. The headspace detection limit for methane was 0.005%, vol/vol.

Batch and Flow-through Column Systems. For batch studies, 43 mL amber vials capped with mininert valves were filled with 20 mL of medium containing 1 mL inoculum from groundwater or wastewater, and were incubated anaerobically with a N₂ headspace. For the

chloroform study, a medium containing 20 mM methanol plus 20 mM acetate or 17 mM butyrate was used to stimulate methanogenesis (Widdel, 1986), and the inoculum was obtained from the Jake Hands wastewater treatment plant in Las Cruces, New Mexico, from the secondary anaerobic digester. For the dichloromethane study, medium "337" with 5 mM nitrate was used (Matzen and Hirsch, 1982), and the inoculum was from the Fred Hervey wastewater plant in El Paso, Texas, from the methanol-fed denitrification tank. For the CFC-11 study, medium "337" was used, except 5 mM sulfate was used instead of nitrate, and the inoculum was from an aquifer contaminated with halogenated aliphatic compounds, including CFC-11.

The two-stage anaerobic bioreactor was designed to mineralize chloroform to the nontoxic products of CO₂ and cell biomass. The 30 cm X 3.2 cm columns were packed with 0.7 - 1.0 mm dia. sand, autoclaved twice on successive days and inoculated with 30 mL of 10X concentrated wastewater. The first column was inoculated with the Las Cruces secondary anaerobic sludge and fed upflow (0.1 mL/min) with the same methanogenic medium described above, amended with 0.5 to 2 ppm chloroform. The second column was inoculated with the El Paso denitrification tank fluid and fed with "337" medium with 5 mM nitrate, amended with 4 to 17 ppm dichloromethane. The columns had an approximate residence time of approximately 6.6 hours. Chloroform and DCM concentrations were determined by extracting aqueous samples from the column with hexane and analyzing these by gas chromatography using the ECD. The columns were run separately for 113 days (column 1) or 73 days (column 2) to equilibrate the microbial communities and the dehalogenation reactions. The effluent from the first column (containing DCM) was then fed as the influent to the second column. The first column was amended with 20 mM acetate and methanol, and the second column was amended with nitrate and methanol (5 mM each). Sterilized abiotic columns run in parallel showed less than 10% decreases in chloroform and DCM concentrations.

DNA Analyses. The cloned dichloromethane dehalogenase (*dcm A*) and Cu-type nitrite reductase (*nir U*) genes were kindly provided by T. Leisenger (LaRoche and Leisenger 1990) and J. Tiedje (Ye et al. 1993); the heme-type *nir S* had been isolated previously by G.B. Smith

and a 0.7 kb *Dde* I fragment of the gene was used (Smith and Tiedje 1992). The probe DNA was labelled with ^{32}P labelled d-CTP using the random primer reaction. Genomic DNA was vacuum-filtered (slot blots) or capillary transferred (Southern) onto nylon membranes ("Zeta Probe", Bio-Rad Laboratories). The membranes were hybridized with the probes for 16 h at 65°C and were washed at 65°C in 1% SDS, 1 mM EDTA, 40 mM Na_2HPO_4 before exposure for 16 hours on X-ray film. Using this washing procedure and calculating the T_m of the hybrid (Sambrook et al. 1989), only target DNA which had >89% homology to the probes hybridized to reveal the visible bands shown in Figure 5.

RESULTS AND SIGNIFICANCE

Project 1. Biological Dechlorination of Chloroform to Dichloromethane

The chloroform dechlorination study was initiated with 0.3 ppm chloroform in batch cultures using a 1% inoculum of secondary anaerobic sludge from the Las Cruces, NM wastewater treatment plant. Within the first three weeks, chloroform was dechlorinated by the methanogenic consortium when either butyrate or acetate plus methanol was used as the primary carbon and energy sources (Figure 1). After the first three weeks acclimation, dechlorination rates increased. In terms of chloroform dechlorination rate and product formation, there were no obvious differences between these two cultures, both producing nearly stoichiometric levels of dichloromethane (DCM). In an effort to force further DCM dechlorination, no chloroform was added between days 50 and 100, but no significant DCM degradation was observed (Figure 1).

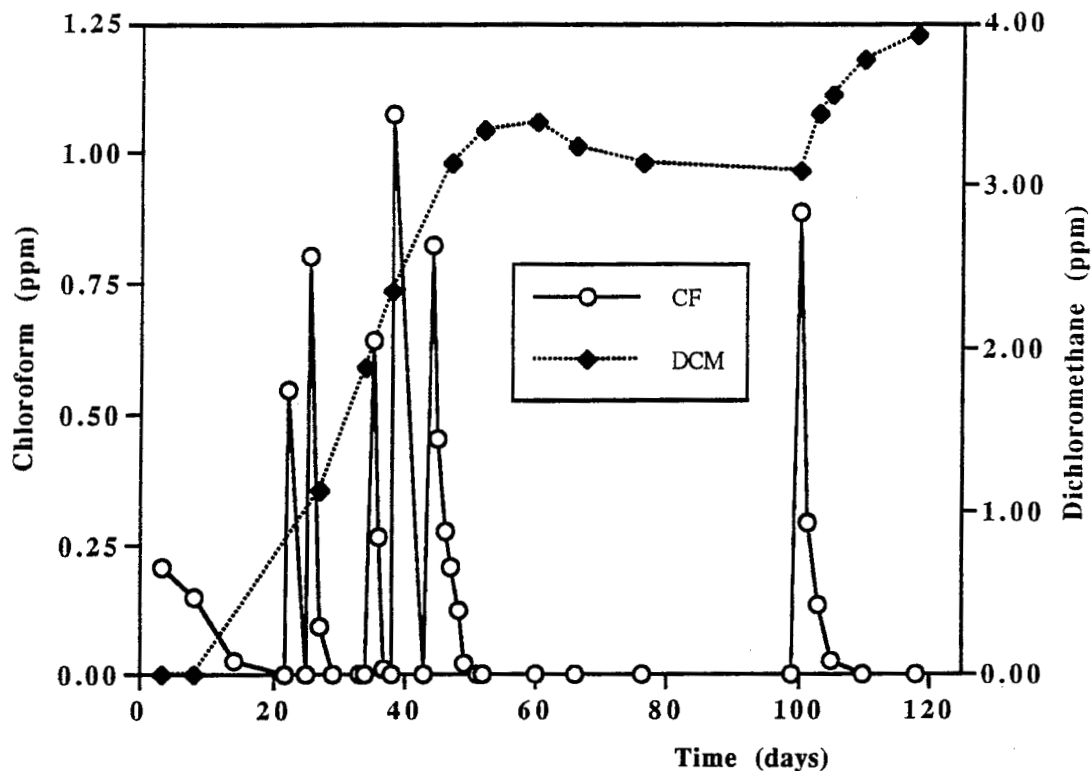


Figure 1. The reductive dechlorination of chloroform (CF) and the corresponding dichloromethane (DCM) formation in a methanogenic consortium sample supported by acetate plus methanol. When butyrate alone was used as the primary carbon source, similar data were obtained. On day 21, 0.5 ppm chloroform was spiked in, and from day 23, 1 ppm chloroform was spiked in as indicated in the figure. Chloroform levels in sterilized controls remained constant over 120 days.

In a study examining the effect of chloroform on the methanogenesis in this mixed culture, no methane production occurred until chloroform levels were biologically reduced to below the gas chromatographic detection limit of 1 ppb chloroform (Figure 2). We observed chloroform concentrations as high as 25 ppm and as low as 10 ppb to completely inhibit methanogenesis until the chloroform was dechlorinated. In contrast, methanogenesis proceeded at similar rates in the presence and absence of 4 ppm DCM, except that in the presence of DCM, the lag period in methane production doubled from 12 to 24 days (data not shown). These data demonstrate that the methanogens present in this sample are extremely sensitive to low levels of chloroform.

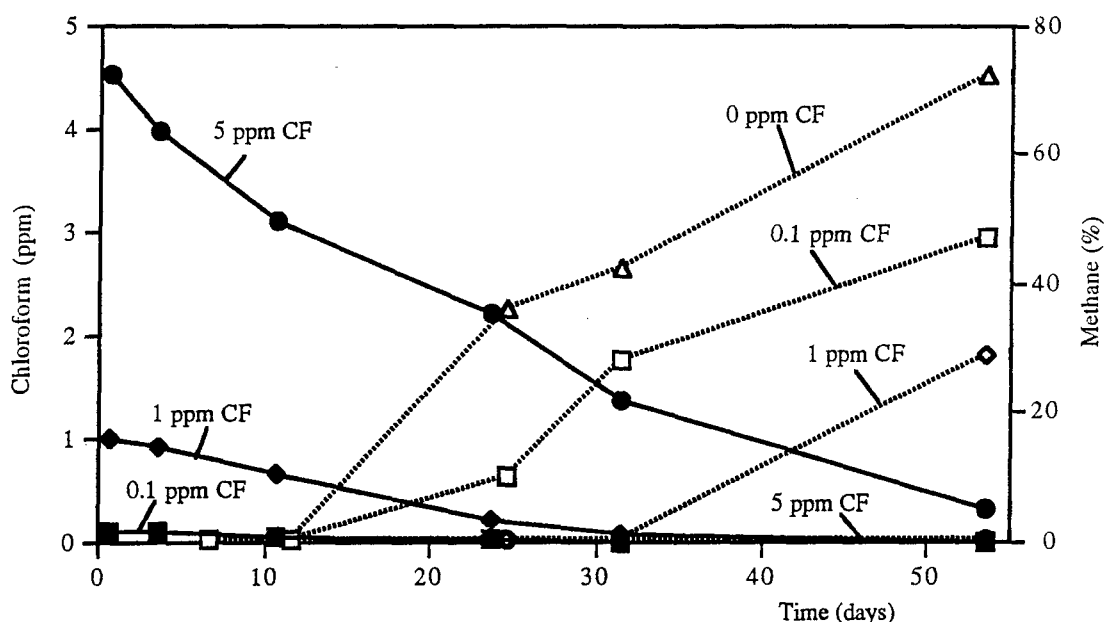


Figure 2. Chloroform dechlorination (closed symbols) and methane production (open symbols) in the presence of varying concentrations of chloroform.

To determine what physiological group of bacteria were active in degrading chloroform, a specific inhibitor of methanogenic bacteria, bromoethanesulfonic acid (BES), was used. Increasing concentrations of BES increasingly inhibited both dechlorination and methanogenesis, with methanogenesis being more sensitive to BES than dechlorination (Figure 3). After 25 days of incubation, the BES concentration at which 50% inhibition was observed (IC_{50}) was 0.44 mM BES for chloroform dechlorination and 0.06 mM BES for methanogenesis. Interestingly, the BES inhibition of methanogenesis and chloroform dechlorination was significantly reversed by addition of coenzyme M (CoM) when increasing levels of CoM were added to the methanogenic cultures (Figure 4). Coenzyme M is a coenzyme unique to methanogens involved in the final methyl reduction stage of methanogenesis, and BES is an analog of coenzyme M. The BES inhibition, and the CoM reversal, of chloroform dechlorination indicate that methanogenic bacteria are the major dechlorinating bacteria present in the sample. Of course there are anaerobes other than methanogens present in this sample which may be involved in dechlorination, but a nonmethanogenic reaction which is stimulated by coenzyme M would be unusual.

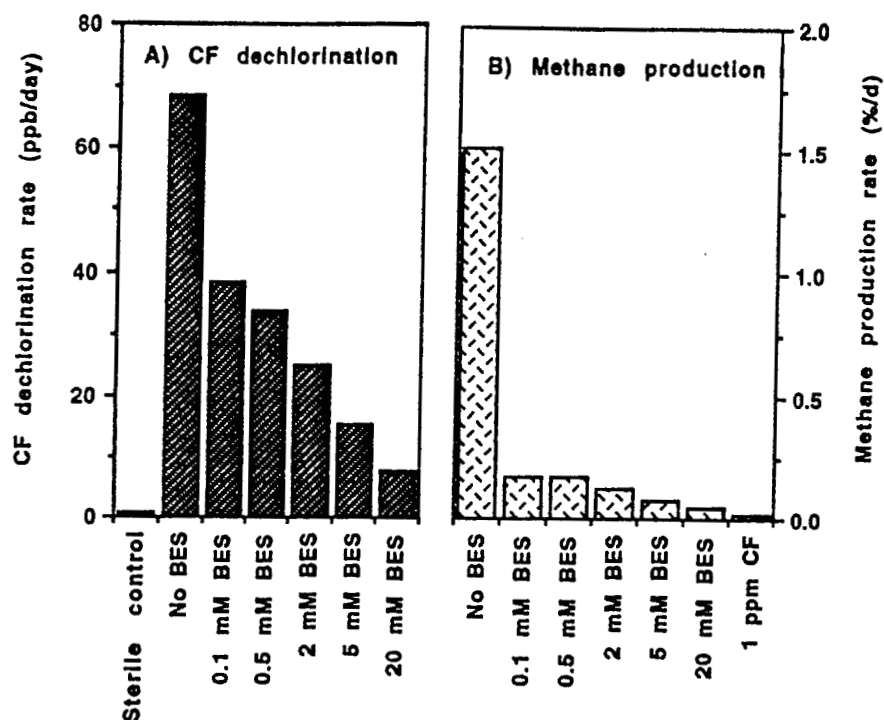


Figure 3. Inhibition of chloroform (CF) dechlorination (A) and methanogenesis (B) by BES. The data shown here are calculated after 25 days incubation. The chloroform concentration was 1 ppm. Because chloroform inhibited methanogenesis, the inhibition study of methanogenesis by BES was carried out in the absence of chloroform.

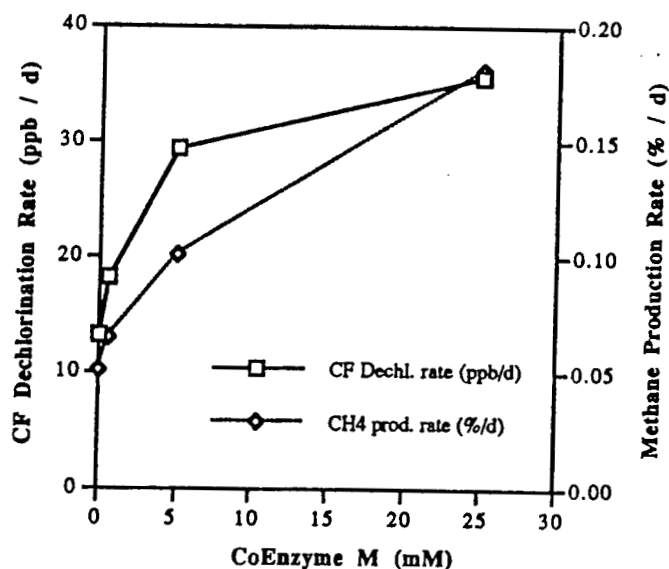


Figure 4. Reversal of BES inhibition of chloroform (CF) dechlorination and methanogenesis by addition of increasing concentrations of coenzyme M (CoM). The BES concentration was 5 mM and the data was taken after 25 days incubation. The methanogenesis study was carried out in the absence of chloroform.

In this work, we are proposing that the moiety which is the target of chloroform toxicity, is also the catalyst by which the chloroform is detoxified to DCM (Yu and Smith, 1995, 1996a). The product of the reaction, DCM, is approximately 1000 times less inhibitory to methane production than the substrate, chloroform (Yu and Smith, 1996). It has been known for some time that chloroform inhibits methanogenesis in anaerobic digestors, and the toxicity can disrupt this critical stage of wastewater treatment (Stickley, 1970; Bagley and Gossett, 1995). Yang and Speece (1986) have observed that chloroform toxicity on methanogenesis was reversible once chloroform was removed abiotically from the system. Our work has advanced these studies in that we have evidence showing how the chloroform inhibition of methanogenesis is related to the detoxification reaction. We are in the process of identifying the particular methanogenic reactions and catalysts responsible for these effects. To study the means by which chloroform is dehalogenated, is also to study the means by which chloroform is detoxified at this critical stage of wastewater treatment.

Project 2. Dichloromethane Degradation by a Denitrifying Wastewater and a Pure Culture of *Hyphomicrobium*--Correlation of Enzyme Activity and Genetic Potential

Cultures of *Hyphomicrobium* strain X degraded DCM under denitrifying conditions to below detection limits after 15 days in the presence of methanol and after 21 days in the absence of methanol. After additional amounts of DCM were added, degradation rates were considerably faster (Figure 5A). In contrast, the wastewater sample degraded DCM only after a lag time of between 70 and 100 days (Figure 5B). At day 86, nitrate was assayed for using the diphenylamine test, and was still present in the live incubations (data not shown). As observed with pure cultures of *Hyphomicrobium* strain X, DCM degradation in the wastewater sample was independent of the presence of methanol. This observation of long lag times before DCM degradation was observed has been repeatedly observed with separate samples from this wastewater denitrification tank (Yu et al. 1996).

Figure 5

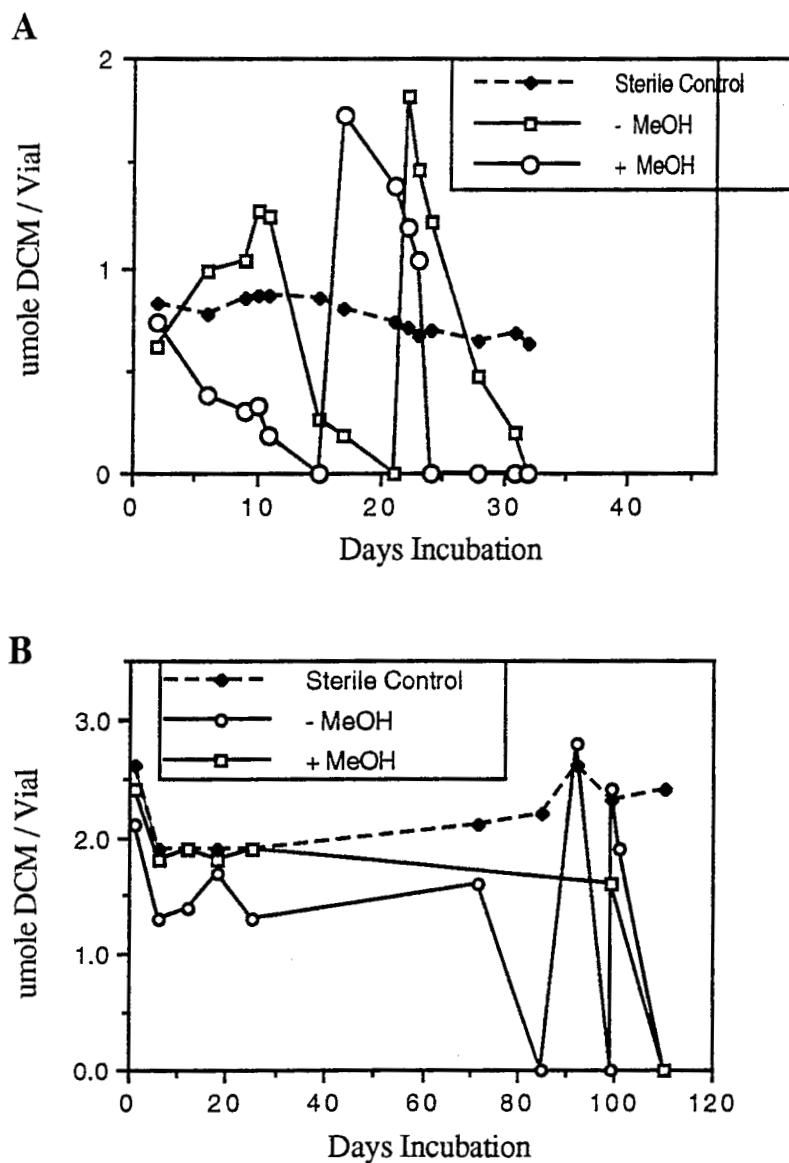


Figure 5. Dichloromethane (DCM) degradation by **A.** *Hyphomicrobium* strain X and **B.** wastewater sample in the presence and absence of 10 mM methanol. In **Figure A.** on days 17 and 22, 0.8 umole DCM was added to the plus- and minus-methanol *Hyphomicrobium* cultures respectively. In **Figure B** on days 92 and 99 ca. 2.5 umole DCM was added to the minus-methanol wastewater sample.

A gene probe specific for DCM dehalogenase (*dcm* A) hybridized to DNA extracted from the wastewater microbial community (Figure 6, lanes 1A & 1B) and to DNA from *Hyphomicrobium* strains X and 81.6 (lanes 1D & 1E). Interestingly, the DCM dehalogenase gene was present in the original wastewater sample (lane 1A), and then was selected for during enrichment to yield much higher copy numbers (lane 1B); this was a separate enrichment experiment than the one shown in Figure 5. As shown above in Figure 5B, consistent DCM degradation activity in this sample was only observed after 70 to 100 day lag periods, yet *dcm* gene probe results show that the gene was indeed present in the original sample. We believe this is an example of the problem that culturing environmental microorganisms introduces; many microbes do not grow or express enzyme activity during laboratory incubation, yet have the genes necessary to do so, and so are not easily measurable by culture-based techniques. These results show the benefit of using genetic techniques which assay directly for the gene, circumventing the bias introduced when assaying for enzyme activity. A positive gene probe result can be used to improve incubation conditions to exploit the enzyme degradation activity for remediation purposes.

Both gene probes specific for denitrifying bacteria (*nir* S is the heme-type and *nir* U is the copper-type nitrite reductase) hybridized to the wastewater samples, but after enrichment only the heme-type *nir* probe hybridized, indicating the selection for the heme-type over the copper-type denitrifiers (Figure 6, lanes 2A, 2B, 3A, 3B). None of the species of *Hyphomicrobium* hybridized to either of the denitrifier probes, indicating that they harbor a novel enzyme for reducing nitrite--previously, all denitrifying bacteria tested to date have harbored either the heme-type or copper-type nitrite (Coyne et al. 1989; Smith and Tiedje, 1992; Ye et al. 1993).

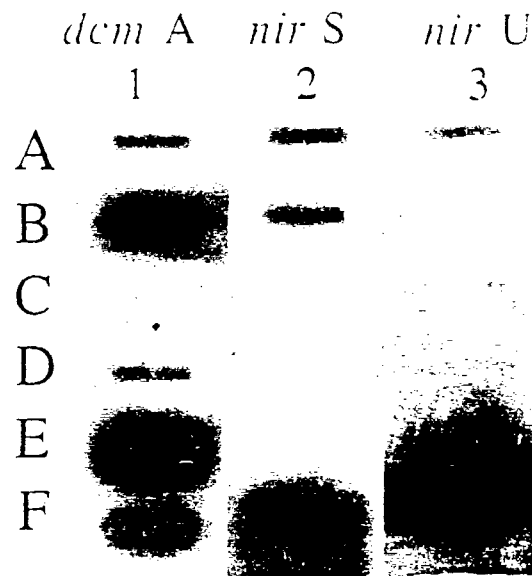


Figure 6. DNA from wastewater biomass (A & B) and from *Hyphomicrobium* strains (C, D & E) was exposed separately to 3 probes (1, 2 & 3). Figure shows the slot blot hybridization of DNA isolated from an El Paso, Texas wastewater sample (row A is DNA from the original wastewater sample, row B is DNA from the sample after enrichment in the presence of DCM) and of DNA from pure cultures of *Hyphomicrobium* (row C = strain T-37, row D = strain 81.6 and row E = strain X). The samples were probed with 1. *dcm A* (coding for the DCM dehalogenase enzyme), 2. *nir S* or 3. *nir U* (*nir* = the gene for the nitrite reductase enzyme from denitrifying bacteria; *nir S* is the heme-type and *nir U* is the copper type nitrite reductase gene. Row F has the respective positive control for each of the probes, (with the *nir U* control overloaded).

Project 3. Design and Function of a Two-stage Anaerobic Bioreactor to Degrade Chloroform

As we have reported in Project 1 (Yu and Smith 1995, 1996a), chloroform degradation under methanogenic conditions commonly results in the accumulation of dichloromethane (Bagley and Gossett 1995). In an effort to integrate the results of Projects 1 and 2, we have designed a 2-stage bioreactor to dehalogenate chloroform sequentially to CO₂ and cell biomass (Yu and Smith, 1996b). It is not uncommon for bioengineers to design a sequential anaerobic-aerobic 2-stage bioreactor to dehalogenate highly halogenated compounds anaerobically, the products of which can be fed into an aerobic reactor to further dehalogenate and mineralize the compound (eg. Fathepure and Vogel, 1991). Aeration is the most common way to deliver oxygen to reactors but a limitation is that this also is an efficient way to air-strip volatile pollutants such as chloroform and DCM. To address this problem and to exploit the DCM degradation activity observed under denitrifying conditions reported in Project 2 (Melendez et al. 1993; Yu et al. 1996), we have designed a chloroform-degrading bioreactor in which both stages are kept anaerobic (Yu and Smith, 1996b).

As described in the Methods section above, we inoculated two columns with organisms from the Las Cruces primary digester or from an El Paso denitrification tank. The first column was maintained under methanogenic conditions and was able to degrade chloroform to DCM, and the second column was maintained under denitrifying conditions and was able to degrade DCM. Since the *dcm A* gene was amplified many-fold during enrichment of this sample in the presence of DCM (Figure 6), and since we have not measured chloromethane even transiently produced, we assume the DCM is degraded to CO₂ and biomass via the DCM dehalogenase pathway (Kohler-Staub and Leisinger, 1985). Abiotic columns with sterilized inocula degraded, sorbed or volatilized less than 10% of either chloroform or DCM. After equilibration, the two live columns were connected in series and chloroform was fed to the influent of the methanogenic column. The effluent of this column, containing DCM only, was amended with 5 mM nitrate and 5 mM methanol. For the next 60 days, greater than 99% of the chloroform was degraded and between 90

and 100% of the DCM was degraded (see Figure 7 as an example of data taken during this period). Over the next 6 months of continuous operation, the performance of the 2nd denitrifying column decreased, with only 50 - 60 % of the DCM being degraded. However, chloroform was never measurable in the column effluents, showing the stability of the methanogenic dechlorination process. Future work with this novel 2-stage anaerobic bioreactor will involve the re-establishment of DCM dehalogenation in the second column and the optimization of the system to be run on a single carbon source (methanol).

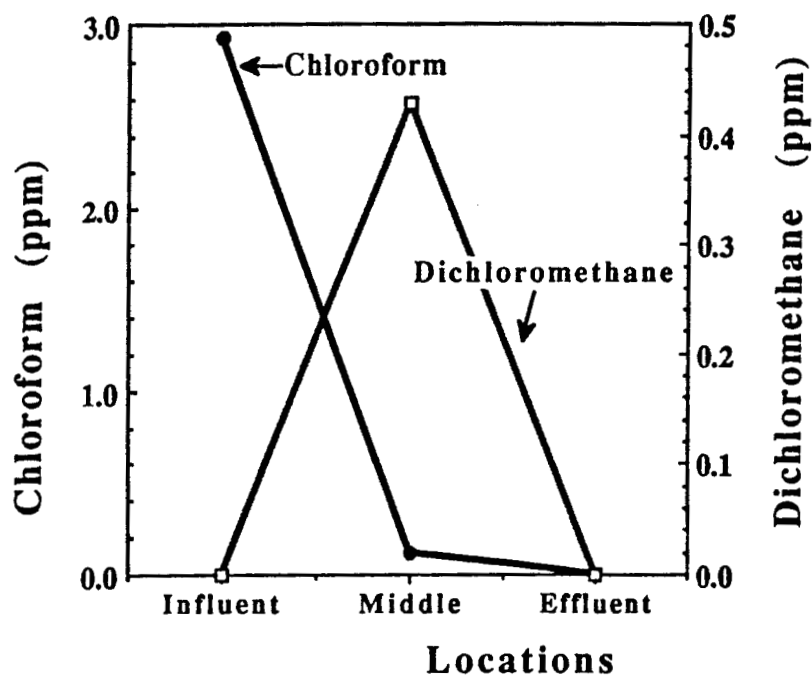


Figure 7. Three ppm chloroform was delivered to a two-stage anaerobic bioreactor designed to sequentially treat chloroform to dichloromethane under methanogenic conditions, and dichloromethane to CO₂ under denitrifying conditions. Shown in this figure are the concentrations of chloroform and dichloromethane from the influent of the first methanogenic column, from the middle of the two columns, and from the effluent of the second denitrifying column.

Project 4. Dehalogenation of Chlorofluorocarbons (CFCs) by Aquifer Bacteria

Bacteria sampled from an aquifer contaminated with halogenated aliphatic compounds were shown to dechlorinate CFC-11 (trichlorofluoromethane) to HCFC-21 (dichlorofluoromethane) (Sonier et al. 1994). The process was shown to be dependent on the presence of both an electron donor (acetate, propionate or butyrate, but not formate) and an electron acceptor (sulfate, thiosulfate or elemental sulfur), and was inhibited by molybdate, an inhibitor of sulfate-reducing bacteria (Duran and Smith 1996). We have shown this aquifer enrichment culture to be able to reductively remove a single chlorine group from chloroform and CFC-113 (1,1,2-trichlorotrifluoroethane)--the identities of these dechlorination products were verified by mass spectrometry (Duran and Smith, 1995).

Other researchers have shown CFC-11 degradation to occur extracellularly in the presence of heme or corrinoid cofactors (Lovley and Woodward, 1992; Krone et al. 1991). However, never has any of our sterilized controls abiotically degraded CFC-11 (Sonier et al. 1994; Duran and Smith 1995). Recently we have performed an experiment to test whether the activity is cell-associated by removing the cells from an actively degrading culture and assaying for CFC-11 degradation in the different fractions (Figure 8). Results verify that the activity is not an extracellular process, since the cell-free filtrate lost the activity but the fraction containing cells continued dechlorinating CFC-11 (Figure 8).

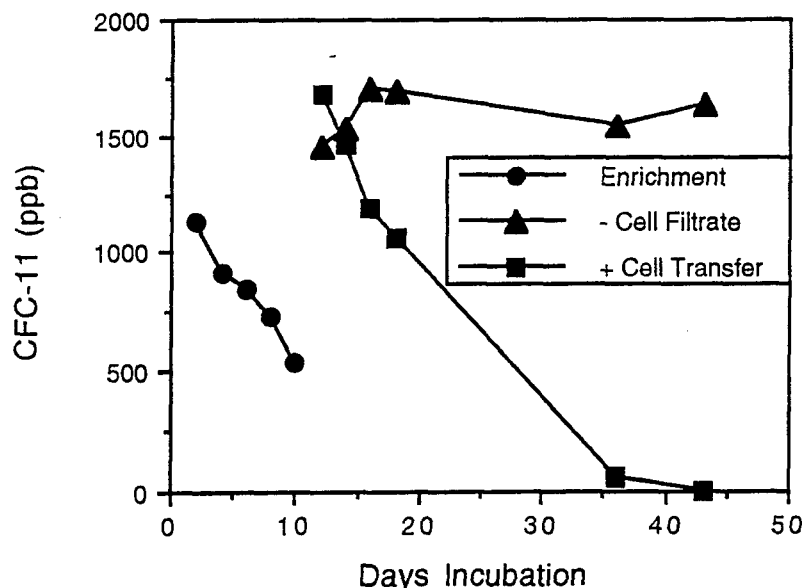
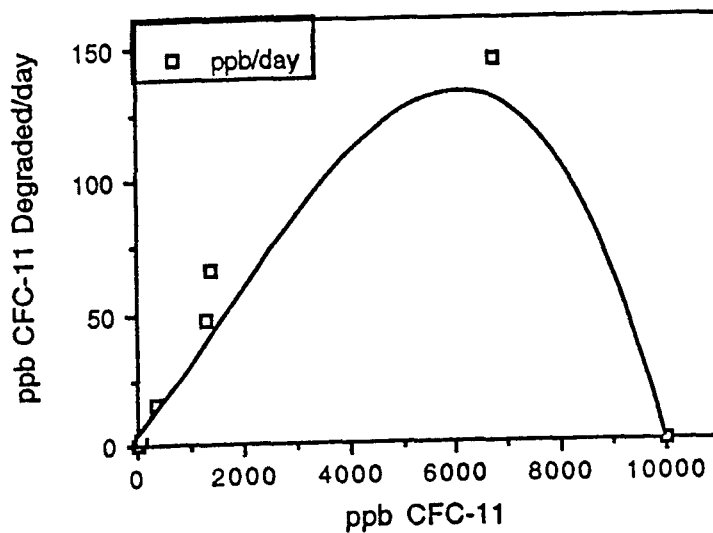


Figure 8. An actively dechlorinating enrichment culture of aquifer bacteria was filtered on day 10 through a 0.2 μm nylon filter in order to test which fraction was responsible for CFC-11 dechlorination. The minus-cell filtrate lost activity whereas the fraction containing cells continued dechlorinating at the original rate. To ensure anaerobiosis, filtration was carried out under a sterile stream of nitrogen gas.

An intriguing observation in this CFC dechlorination work has been the biphasic rate dependence on the concentration of CFC-11 substrate (Figure 9). Following Michaelis-Menten kinetics, the dechlorination rate increases in response to CFC-11 concentration, except that between 7 and 10 ppm CFC-11, the rate drops to zero (Figure 9A). This inhibition at levels of CFC-11 above 5 to 7 ppm has been repeatedly observed in four separate experiments (Duran and Smith, 1996). At low concentrations (ca. 10 ppb), we have repeatedly observed no degradation by the bacteria (Figure 9B for example). This indicates that these low levels of CFC-11 are biologically unavailable to the dehalogenating bacteria, and has important implications regarding pollutant concentrations below which bioremediation may not be feasible.

Repeated attempts to isolate the CFC-11 degrading bacterium from this enrichment have not been successful to date. Two pure cultures have been isolated but neither dechlorinates CFC-11. Further isolation work is being carried out using filtration to separate the bacteria by size--what we think is the CFC-11 degrading bacterium forms diplobacilli which are up to 8 μm in length.

A.



B.

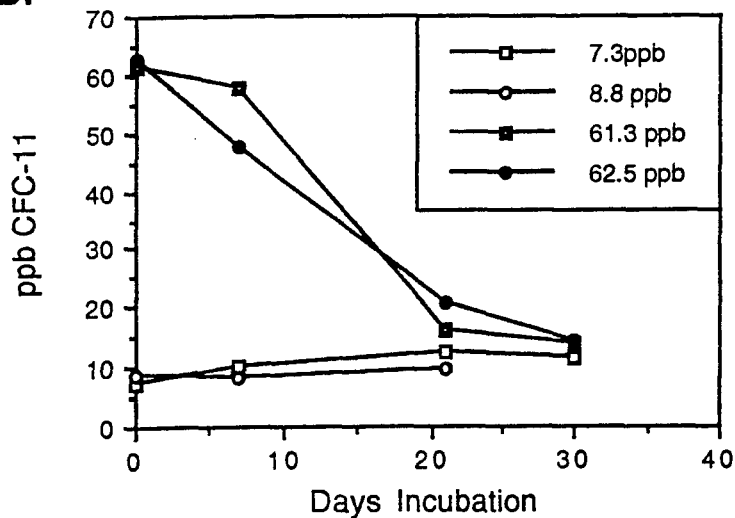


Figure 9. CFC-11 dehalogenation data from aquifer sulfate-reducing bacteria. **A.** = the rate response between 0 and 10 ppm CFC-11 showing no degradation at the two extremes of low or high CFC-11. **B.** = at low concentrations of CFC-11 of ca. 10 ppb, no degradation was observed. Note that the concentrations of CFC-11 shown are the nominal concentrations--See the Methods section for discussion of Henry's Constants.

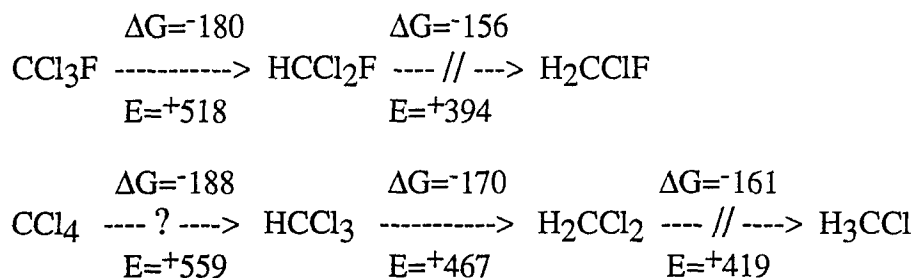
Project 5. Use of Thermodynamic Parameters to Predict Which Dehalogenation Reactions are Favored

Toward a thermodynamic understanding of the CFC dehalogenation reaction, I have begun a collaboration with Professor Jan Dolfing (Research Institute for Agrobiological Sciences, Haren, The Netherlands) in which we have performed thermodynamic calculations of chlorofluoromethane reductive dehalogenation reactions. Using the CFC-11/HCFC-21 redox couple as an example, we have shown that it is highly oxidized, having a redox potential of +518 mV. CFC-11 dechlorination would therefore be a thermodynamically superior electron acceptor compared to nitrate/nitrite (+420 mV) and sulfate/H₂S (-220 mV), for example. Using ΔG_f° values from Lange's Handbook of Chemistry (1985), we have calculated the reductive dechlorination of CFC-11 to HCFC-21 to be highly exergonic, with a $\Delta G^\circ = -179.94$ kJ/mol. These thermodynamic calculations predict that the single dechlorination of CFC-11 is the most energetically favorable reaction compared to a defluorination event for example. The predictions fit both our observed dechlorination patterns (Sonier et al. 1994; Duran and Smith, 1995) and those of Lesage et al. (1990), thus indicating that these type of calculations are applicable to in situ groundwater reactions.

The dehalogenation substrate and products shown in Table 1 have been verified by mass spectrometry to be mediated by a sulfate-reducing enrichment culture originally from an aquifer contaminated by halogenated aliphatic compounds (Sonier et al. 1994; Duran and Smith, 1995). None of these reactions were observed in sterilized controls. What is extremely interesting about these reactions is that a C-1 hydrochlorofluorocarbon, HCFC-21 (HCCl₂F), with an oxidation state of +2 was not dehalogenated and yet the chlorinated equivalent, HCCl₃, was dehalogenated further to its reduction product, H₂CCl₂. We propose that these series of reactions span a critical ΔG and redox level which could be used to predict whether dehalogenation will occur under sulfate-reducing conditions. From the above reactions, these threshold values are between a ΔG of -161 and -169 kJ/mol and a redox between +419 and +466 mV.

Table 1. Reductive dehalogenation reactions of CFC-11 (CCl_3F) and chloroform (HCCl_3) mediated by an aquifer enrichment of sulfate-reducing bacteria (Dolfing and Smith, manuscript in preparation).

Oxidation State
of Carbon+4.....+2.....0.....-2.....



$\Delta G = \Delta G^\circ$ (kJ/mol) and $E = E^\circ$ (mV). Both were calculated as described above.

// = Reaction was tested and not observed to proceed.

? = Reaction to be tested.

PRINCIPAL FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

Biological dehalogenation of trichloromethane (chloroform), dichloromethane (DCM) and trichlorofluoromethane (CFC-11) was identified under the respective reducing conditions of methanogenesis, denitrification and sulfidogenesis. In our work with wastewaters we have shown that the chloroform-degrading wastewater cannot degrade DCM, and the DCM-degrading wastewater cannot degrade chloroform. These observations fit well a thermodynamic interpretation : chloroform is oxidized enough to be dehalogenated under highly reducing conditions such as methanogenesis, but under denitrifying conditions there is not enough reductant available to reduce chloroform. Instead, under denitrifying conditions, DCM can be used as a carbon and energy source since there is energy available to microbes for oxidizing DCM to CO_2 . A direct application of these results is the use of a novel 2-stage anaerobic bioreactor which sequentially degrades chloroform to DCM and DCM to CO_2 and biomass.

When we assayed the denitrifying wastewater sample for the potential to degrade DCM, gene probe results, within one week of sampling, indicated the presence of the gene coding for a

DCM dehalogenase enzyme and thus demonstrated that the wastewater microflora could be capable of DCM detoxification. In contrast to this molecular genetic approach, the more traditional technique of monitoring the sample for DCM degradation activity gave inconsistent results and only after 3 months of incubation. Thus, the use of gene probes specific for pollutant biodegradative genes can be a more efficient method to show the feasibility of bioremediating a water environment, compared to techniques which require environmental microbes to grow in laboratory incubations. Use of gene probes to identify the potential for bioremediation is being developed in our laboratory and others (Yu et al. 1996; Fleming et al. 1993).

The environmental impact of the CFC dechlorination reactions is apparent when one considers that CFC-11 has the greatest potential for ozone destruction among the commonly used CFCs (Wallington et al. 1994) and has a global warming potential 1300 times that of CO₂ (Lashof and Ahuja, 1990). The single dechlorination step observed here has reduced a CFC into an HCFC (hydrochlorofluorocarbon), greatly diminishing the potential for ozone degradation and global warming. The CFCs are present as groundwater contaminants at concentrations ranging from 0.1 to 2 ppm and thus are in the concentration range amenable for biological dechlorination. It may be advantageous to stimulate CFC biological dechlorination before the CFCs eventually escape into the atmosphere, as was first suggested by Lovley and Woodward (1992). CFC dechlorination could be stimulated by aquifer amendment with acetate--there are plenty of sulfates, 200-400 ppm, in this groundwater (Sonier et al. 1994).

Finally, the use of thermodynamic parameters associated with dehalogenation reactions (such as the change in free energy and redox) can allow for the prediction of which dehalogenation reactions are favored to occur under what conditions. Because of the collaborative help of J. Dolfing, these calculations can now be applied to CFC dehalogenation. The calculation and use of these parameters will greatly help in showing whether bioremediation of halogenated aliphatic compounds is feasible given environmental measurements such as dissolved oxygen and nutrient status of the water environment.

SUMMARY

Table 2. Summarized below are the dehalogenation reactions reported here and identified in water samples from different environments under three redox conditions.

Site	Redox (e- acceptor)	Reaction	Reference
Tertiary Wastewater	NO_3^-	$\text{H}_2\text{CCl}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_2\text{O} + 2\text{HCl}$	Melendez et al. 1993 Yu et al. 1996
Aquifer	SO_4^{2-}	$\text{CCl}_3\text{F} + \text{H}_2^* \rightarrow \text{HCCl}_2\text{F} + \text{HCl}$	Sonier et al. 1994
Secondary Wastewater	CO_2	$\text{HCCl}_3 + \text{H}_2^* \rightarrow \text{H}_2\text{CCl}_2 + \text{HCl}$	Yu and Smith 1996a

The dechlorination products HCFC-21 (HCCl_2F) and DCM (H_2CCl_2) have been verified by mass spectrometry. The dechlorination product, formaldehyde (CH_2O) has not been identified and is hypothesized after Kohler-Staub and Leisinger, 1985.

* $\text{H}_2 = 2\text{H}^+ + 2\text{e}^-$. The electron donor for the CFC-11 and chloroform reductions is acetate.

The reactions shown in Table 2 span different reduction potentials and thus different mechanisms of dehalogenation are active. Under denitrifying conditions we have shown the presence of the *dcm* A gene, indicating that the dichloromethane is undergoing hydrolytic dehalogenation to formaldehyde which is oxidized rapidly to formic acid and then carbon dioxide, as has been observed by Kohler-Staub and Leisinger (1985). The CFC-11 and chloroform dechlorination reactions are reductive, with the respective end-products identified by mass spectrometry (Sonier et al. 1994; Duran and Smith, 1995). Taken together these samples and respective dehalogenation activities represent differing environments, both natural (albeit contaminated aquifer) and human-dominated (wastewater) water systems and has allowed for an integrative study of the biological dehalogenation reaction.

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